AD_____

Award Number: W81XWH-06-1-0684

TITLE: Estrogen Mobilizes Circulating Bone Marrow Progenitor Cells to Promote Tumor Neovasculature: Lessions from Ischemic Model Provide a Novel Breast Cancer Target

PRINCIPAL INVESTIGATOR: Raj K. Tiwari, Ph.D.

CONTRACTING ORGANIZATION: New York Medical College
Valhalla, NY 10595

REPORT DATE: September 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2007	Final	1 SEP 2006 - 31 AUG 2007

4. TITLE AND SUBTITLE
Estrogen Mobilizes Circulating Bone Marrow Progenitor Cells to Promote Tumor Neovasculature: Lessions from Ischemic Model Provide a Novel Breast Cancer Target

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0684

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Raj K. Tiwari, Ph.D.

E-Mail: raj_tiwari@nymc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

New York Medical College
Valhalla, NY 10595

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Breast cancer growth and metastases is dependent on neovasculature. The cells that actually trigger the formation of new blood vessels are poorly characterized but it has been hypothesized that some of the precursor blood vessel cells originate in the bone marrow and then home to tumor tissues. Although estrogen is a major stimulus, its role as a bone marrow originating endothelial cell mobilizing agent has not been demonstrated. We propose to test the proposition whether estrogen can mobilize circulating bone marrow derived progenitor endothelial cells (EPCs) to the implanted tumor using Green fluorescence protein, GFP, tagged EPCs. The objectives of the proposed study are two fold: A) Establishment of proposed Animal model using normal and OVX (ovariectomized) female BALB/c mice and examine if estrogen can indeed mobilize the EPCs for the generation of new blood vessels. B) To elucidate the contribution of E2-mobilized, BM-derived EPC in promoting neo-vascularization in implanted breast tumor. The mechanism of E2-induced mobilization of EPC from the bone marrow will open a new area of BrCa research and hence novel targets. Moreover, BM-EPCS could potentially serve as the "Trojan horse" to deliver bio-molecules that disrupt tumor vasculogenesis and/or induce targeted killing of tumor cells. Thus this novel basic concept has clinical potential.

15. SUBJECT TERMS
Progenitor stem cells, estrogen, animal model, breast cancer, angiogenesis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	21	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

Introduction:

Neo-vascularization or angiogenesis is critical in sustaining growth of tumors as well as subsequent metastases (1,2). Circulating bone marrow derived endothelial progenitor cells (BM-EPC) have been observed to contribute to neo-vascularization of tumors, especially in lung cancer (2,3,4). The search for molecules which can mobilize BM-EPC and subsequently lead to neo-vascularization of tumors led to the discovery of estrogen (E2) as a potent stimulus (5). With this in mind and provided that the majority of breast cancers are estrogen sensitive, it is conceivable to speculate the estrogen plays a significant role is sustaining the growth of breast cancers through neo-vascularization. To date, no studies have been undertaken to elucidate the mechanism of estrogen in the neo-vascularization of breast cancer. With this in mind, the working hypothesis of this project is that estrogen mobilizes circulating BM-EPC which home to the implanted breast carcinomas and promote tumor neo-vascularization.

Body:

Objective A (from SOW): Establishment of the Tie2-GFP bone marrow deficient mouse model

Tumor incidence and histology. Three groups of female mice (6-8 weeks old) were used for the animal studies. Intact or non-ovaretcomized Tie2-GFP mice (group A), ovarectomized (group B), and ovarectomized + E2 (group C). One week prior to surgery, one group of ovarectomized mice were implanted with 60 day slow release estradiol pellets. At the time of surgery, all mice were anastesized using 0.3cc of Avertine. The fur was completely shaved followed by thoroughly disinfecting the area with 70% alcohol followed by betadine. A small incision was made at the abdomen followed by carefully teasing the skin from the underlying tissue at which time the exposed fourth inguinal mammary glands were implanted with either 2 x 10^6 Tg1 cells/animal (n=3 for all groups) or 4 x 10^6 Tg1 cells/animal (n=4 for all groups). Two experimental endpoints were set up, day 6 and day 12, after which all three groups for both days 6 and12 had a 100% tumor incidence (Table I and II). At the experimental endpoint, tumors were resected along with the corresponding non-tumor bearing fourth inguinal mammary gland as a control. One half of the mammary gland was fixed in 4% paraformaldehyde and subsequently paraffin embedded while the second half was embedded in OCT compound. H&E stained mammary gland sections (40X) from all three groups are in shown in figure 1 in which a loss of normal mammary gland tissue architecture is observed between the control non-tumor bearing gland vs. the tumor bearing gland.

Objective B.I (from SOW): Examining the EPC mobilization into circulation following E2 supplementation and tumor cell implantation

Tg1 mammary tumor neo-vascularization. Detection and visualization of vascular structures or capillaries was through GFP expression and was facilitated by the fact that GFP expression is under the endothelial cell specific Tie-2 promoter. Capillaries were counted by 2 blinded investigators and averaged. A representative tissue section from both non-tumor and tumor bearing mammary glands from all three groups is shown in figure 2A. The average capillary number from six fields is represented in the bar chart in figure 2B. A significant increase in capillaries was observed in intact non-ovarectomized mice between the tumor bearing gland vs. the non-tumor bearing gland. This observation

was decreased in ovarectomized mice. Interestingly, a significant increase ($p<0.001$) in tumor capillary formation was observed in the ovarectomized + E2 group when compared to the ovarectomized group. After nullifying the inter-animal variables and the effect of ovarectomization of all three groups of animals, we observed a 2 fold increase in capillary number in ovarectomized animals supplemented with E2. The mobilization of endothelial cells was further confirmed by immune-fluorescence staining of tumors with CD133 antibody (figure 3). CD133 is a marker of hematopoetic stem cells and the expression is restricted to EPCs and not mature endothelial cells. Comparing all three groups, CD133 positive staining was consistently higher in all three groups when tumor was present. The number of CD133 positive cells was significantly higher ($p<0.001$) in ovarectomized mice that received E2 supplementation (156±2.9 in OVX + E2) compared to ovarectomized mice which did not receive E2 (65±3.1 in OVX). Also, colocalization of CD133 positive endothelial cells with the GFP positive endothelial cells was observed. Co-localization is indicated by the red arrows and appears in yellow. ***These results are clear indicators that E2 mediates neo-vascularization.***

Estrogen supplementation mobilizes BM-derived progenitor cells into circulation. As an initial step to test our hypothesis, the stimulatory effect of E2 on EPCs was investigated. Ovarectomized Tie2-GFP female mice (6-8 weeks of age) were implanted with 60 day slow release estradiol pellets one week pre-surgery. Blood (40µl) was drawn from the tail-vein, both pre and post estradiol pellet implantation, and was subjected to histoplaque density gradient to isolate the mononuclear cells. Mononuclear were then with anti-CD133 antibody and analyzed by flow cytometry. Double positive cells for GFP (endothelial lineage) and CD133 (stem cell marker) were counted as a representative population of circulating EPCs. As shown in figure 4, the number (%) of EPCs in non-estradiol supplemented mice remained at base line (blue) whereas estradiol supplementation significantly increased the number (%) of circulating EPCs, which peaked at day 3 and returned to base line by day 7. ***This data confirms the stimulatory effect of estrogen on EPC mobilization from the BM into circulation*.**

Objective B.II (from SOW): Cytokine profiling of tumor tissue RNA and protein extracts

Endothelial cells secrete paracrine cytokines that presumably affect tissue remodeling and neo-vasculature. It is known in literature that during cardiac injury, endothelial cells not only contribute to the formation of the capillary architecture but also enhance neo-vascularization through the secretion of angiogenic paracrine cytokines such as angiopoetin-1 (Ang1), angiopoetin-2 (Ang2), thrombospondin-1 (tasp-1), vascular endothelial growth factor (VEGF), matrix mettalo-proteases 2 and 9 (MMP2 & MMP9), and basic fibroblast growth factor (bFGF). We wanted to evaluate if this phenemon also exists in our model and to do so, total RNA was extracted and subjected to quantitative real-time PCR (Q-RT-PCR). The transcripts from the angiogenic and tissue remodeling cytokines listed above were evaluated for and the fold increase over the normal mammary gland was plotted in figure 5. As shown in figure 5, E2 supplementation in OVX mice (OVX + E2) consistently and significantly enhanced expression off all tested cytokines indicating that estrogen enhances neo-vascularization.

Development of an in vitro model to examine the proliferative and migratory response of Tg1 tumor cells by EPC secreted cytokines. We independently wanted to evaluate the affect of secreted EPC factors on proliferation and migratory properties of 4T1 tumor cells. To do so, EPCs were cultured for 7 days in the presence or absence of estradiol (10^{-8}M) in complete EBM2 media. The media (both E2 and non-E2 supplemented) from the EPCs was harvested and added to synchronized 4T1 tumor cells for 48 hrs after which the cells were pulsed with tritiated thymidine (1H3-thymidine) for 18 hrs. Thymidine incorporation, indicative of cycling cells, was plotted as average count per minute (cpm) of triplicates. As shown in figure 6A, the media obtained from EPCs supplemented with E2 significantly increased ($p<0.001$) 4T1 cell proliferation (red). Next we wanted to evaluate if the E2 supplemented media from EPCs also contributes to the migration of 4T1 tumor cells using a modified Boyden chamber. As EPC E2 supplemented media increased 4T1 cell proliferation, so too did it increase 4T1 cell migration. As shown in figure 6B, significantly increased ($p<0.001$) numbers of 4T1 cells (red) migrated towards the EPC + E2 conditioned media as opposed to E2 free EPC media (yellow).

E2- induced tumor cell factors modulate EPC function. Lastly, we wanted to determine if tubulogenesis is influenced by E2 *in vitro*. The tubulogenesis, or the formation of tube like structures, is a hallmark activity of EPCs *in vitro*. For this study, EPCs were isolated from intact mouse bone marrow and plated on growth factor reduced matrigel coated dishes in EBM2 media. 4T1 tumor cells were cultured either in the presence or absence of E2 after which the media was harvested and added to the EPC cultures. Tubulogenesis was monitored 24 hrs after the addition of E2 supplemented and non-supplemented 4T1 cell media. As seen in figure 7, untreated control EPC did not display the formation of tube like structures. E2 free EPC media was unable to properly induce tube like structures although a few disorganized tubes were present. Interestingly, E2 supplemented EPC media was able to induce the formation of clearly well-organized and defined tubes.

Key Research Accomplishments:

Our experimental data support our hypothesis and as a result of this funding we were able to establish an animal as well as an in vitro model to examine the contribution of bone marrow derived endothelial cells in neo-vascularization.

- Estrogen supplementation increases Tg1 tumor neo-vascularization in Tie2-GFP mice
- Estrogen supplementation mobilizes BM-derived progenitor cells into circulation
- Endothelial cells secrete tissue remodeling and angiogenic paracrine cytokines
- Tg1 tumor cells migrate and proliferate in response to EPC secreted cytokines *in vitro*
- Estrogen induces tumor cells to secrete factors which modulate EPC function

Reportable Outcomes:

Abstract . Abstract supported by award # W81XWH-06-1-0684 was submitted to the 2008 Era of Hope meeting to be held in the Baltimore Convention Center

Manuscript in preparation. Suraino, R., Johnson, R, Lambers, E., Badithe, A., Kishore, R., Tiwari, R. Neo-vascularization by bone marrow derived endothelial progenitor cells is critical for breast cancer progression and is modulated by estrogens (preparation for Cancer Research)

Animal Model Developed. Tie2-GFP mice were ovarectomized and supplemented with 60-day slow release Estradiol pellets to observe the effect of estradiol on breast tumor neo-vascularization. The choice of using Tie2-GFP mice is because formation of capillaries can easily be monitored by GFP which is under under the endothelial cellspecific Tie-2 promote

Conclusion

The ability for tumors and subsequent metastases to form self sustaining entities through neo-vasculogenesis provides a major hurdle which is difficult to overcome (1). Studies pertaining to neo-vasculogenesis in cardiac ischemic injury and tumor formation demonstrate that circulating BM derived EPC are central to neo-vasculogenesis (2). The search for molecules which mobilize EPCs and promote neo-vasculogenesis, such as estrogen (5), may hold the key as to how breast cancers, of which the majority start out as estrogen responsive, become self sustaining through neo-vasculogenesis. Therefore, the role of estrogen in breast cancer development and sustainment is in need of further evaluation, which we are currently investigating. Taking into consideration all the results obtained and presented in the project thus far, it is clear that **estrogen plays a significant role in neo-vascularization** in Tg1 tumors, which was observed via increased capillary synthesis demonstrated by increased GFP fluorescence and CD133 positive staining cells. The contribution of estrogen in **angiogenesis** and **tissue remodeling**, which are two processes indispensable for the growth of tumors, was also observed through Q-RT-PCR in which the transcripts of various cytokines involved in these two critical processes were significantly increased. Lastly, estrogen stimulated EPCs secreted factors which were able to increase tumor cell proliferation and motility while at the same time induce tumor cells to form tube like structures indicative of EPC function. Therefore, we have demonstrated that indeed estrogen plays a critical role in breast cancer neo-vascularization and this knowledge may help us to develop new anti-estrogens directed against estrogen responsive cancers.

References:

1. Komorova NL, Mironov B. On the role of endothelial progenitor cells in tumor neovascularization. J Theor Biol.;235 :338-49, 2005.

2. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA, Hajjar KA, Manova K, Benezra R, Rafii S. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med., 7:1194-1201, 2001.

3. Losordo DW. Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease: part II: cell-based therapies. Circulation. 109:2692-7, 2004.

4. Kawamoto A, Asahara T, Losordo DW. Transplantation of endothelial progenitor cells for therapeutic neovascularization. Cardiovasc Radiat Med. 3:221-5, 2002.

5. Iwakura A, Luedemann C, Shastry S, Hanley A, Kearney M, Aikawa R, Isner JM, Asahara T, Losordo DW. Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendothelialization after arterial injury. Circulation. 108:3115-21,2003

Appendices

Abstract submitted to the 2008 Era of Hope meeting in the Baltimore Convention Center

Neo-vascularization or angiogenesis is critical in sustaining growth of tumors as well as subsequent metastases. Circulating bone marrow derived endothelial progenitor cells (BM-EPC) have been observed to contribute to neo-vascularization of tumors and the identification of cellular and molecular mediators will impact clinical care. In this pilot program we focused on developing an animal model that could examine the contribution of bone marrow derived progenitor endothelial cells (BM-EPC) on neo-vascularization. Further, since estrogen is a major modulator of breast cancer initiation and progression and that estrogen affects BM-EPC migration, we examined the contribution of the BM-EPC induced neo-vascularization. Our animal model was designed to test the hypothesis

"Estrogen mobilizes circulating BM-EPC which home to the implanted breast carcinomas and promote tumor neo-vascularization." We utilized Tie2/GFP-Balb/c ± ovariectomized ± estrogen supplementation. These mice were transplanted with bone marrow derived from Tie2/GFP mice that were used as donors. Tumors were induced in these mice by surgical implantation of Tg1 or 4T1 (ATCC) murine mammary adenocarcinoma cells (derived from syngeneic BALB/c mice; 2×10^6 cells/0.3 ml PBS) into the fourth inguinal mammary gland after clearing the fat pad region of BMT mice. At the end of the experimental period tumor incidence and progression was monitored by immunohistochemical analysis and the BM-EPCs mobilization at the tumor site was measured and correlated with capillary density. We observed the concomitant mobilization of GFP and CD133 (marker of EPC) double-positive cells at the tumor site. Comparison of estrogen supplemented and non-supplemented group, revealed that E2 supplementation enhances both mobilization of GFP-CD133+ cells (EPCs) in the tumors as well as mobilized EPCs physically integrate into neo-vasculature resulting in significantly higher capillary density. The contribution of estrogen in angiogenesis and tissue remodeling, which are two processes indispensable for the growth of tumors, was also observed through Q-RT-PCR experiments on excised tumor-inoculated mammary tissues, in which the transcripts of various angiogenic cytokines involved in these two critical processes were significantly increased. E2 stimulated EPCs were also observed to secrete paracrine factors which increased the proliferation and migration of 4T1 tumor cells. Lastly, EPCs isolated from BM were observed to form tube like structures (tubulogenesis) upon addition of media obtained from E2 stimulated tumor cells. In conclusion we have discovered a novel role of estrogen stimulated BM-EPCs in tissue remodeling in a breast cancer animal model and presumably a novel therapeutic

Supporting Data:

Table I. Tumor incidence in Tg(TIE2GFP)287Sato/J (6-day)

	Intact Tie2GFP mice (Ctrl)	**Ovarectomized Tie2GFP mice (OVX)**	**Ovarectomized Tie2GFP mice + E2 (OVX + E2)**
2 x 10^6 Tg1 cells/mouse	3/3 tumor bearing mice	3/3 tumor bearing mice	3/3 tumor bearing mice
4 x 10^6 Tg1 cells/mouse	4/4 tumor bearing mice	4/4 tumor bearing mice	4/4 tumor bearing mice

Table II. Tumor incidence in Tg(TIE2GFP)287Sato/J (12-day)

	Wild type Tie2GFP mice (Ctrl)	**Ovarectomized Tie2GFP mice (OVX)**	**Ovarectomized Tie2GFP mice + E2 (OVX + E2)**
2 x 10^6 Tg1 cells/mouse	3/3 tumor bearing mice	3/3 tumor bearing mice	3/3 tumor bearing mice
4 x 10^6 Tg1 cells/mouse	4/4 tumor bearing mice	4/4 tumor bearing mice	4/4 tumor bearing mice

Figure 1

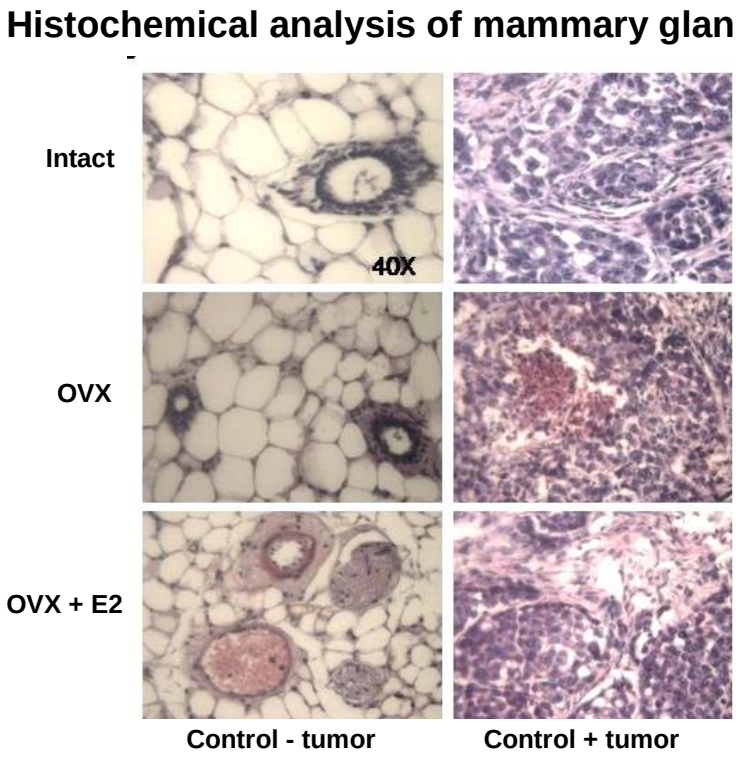

Figure 1. H&E representative figures (40X) from control and tumor cell injected mammary glands cut in a continuous fashion, in Tie2-GFP mice. H&E staining reveals loss of tissue architecture in tumor bearing mice

Figure 2

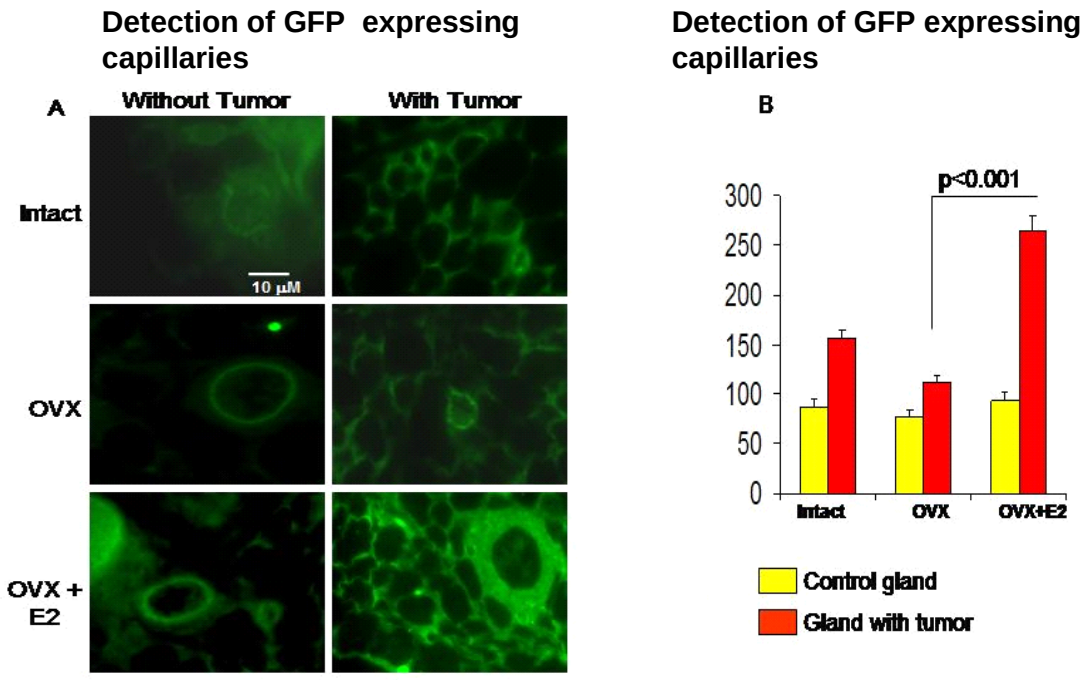

Figure 2. Fluorescence microscopy of fixed (4% paraformaldehyde) mammary glands reveals increased numbers of capillaries in tumors from animals supplemented with estradiol (Ovx + E2) (A). Capillary numbers (6 random fields per section) were counted. The results indicate a significant increase ($p<0.001$) in GFP expressing capillaries in estradiol supplemented ovarectomized (OVX + E2) mice compared to non-estradiol supplemented ovarectomized (OVX)

Figure 3

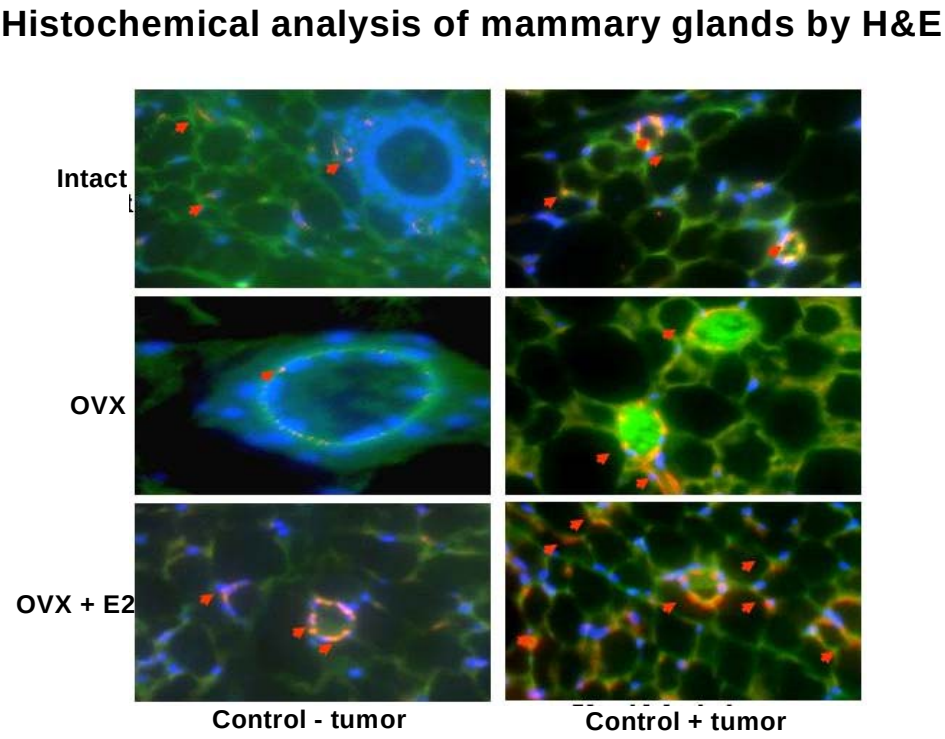

Figure 3. E2-mobilized EPCs home to the sites of tumor and participate in tumor vascularization. Paraformaldehyde fixed mammary gland sections were subjected to immuno-fluorescence staining for CD133 positive cells. The number of CD133+ cells was consistently increased in the mammary glands that were transplanted with tumor cells. The number of CD133+ cells was significantly more in mice that received E2 supplementation (65$\pm$3.1 CD133+ cells in OVX+ tumor samples vs. 156$\pm$ 2.9 CD133+ cells in OVX+E2+tumor samples; $p<0.001$). CD133+ cells co-localized with GFP+ capillaries (indicated by yellow fluorescence and red arrow) suggesting that EPCs not only home to tumor bed but also physically associated with the new blood vessels.

Figure 4

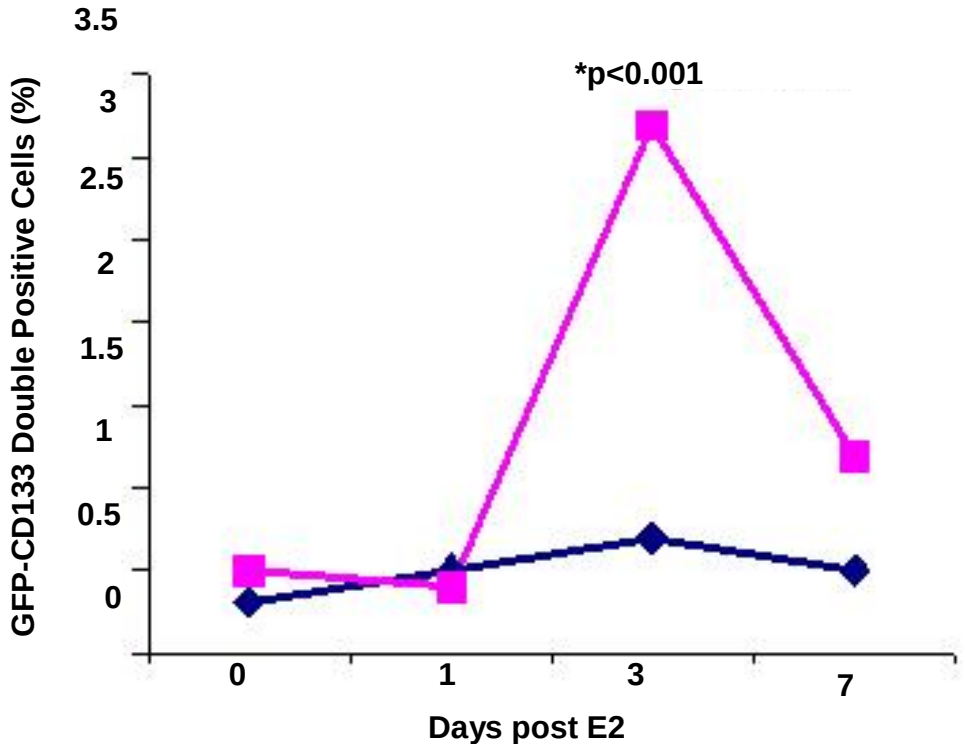

Figure 4. Estrogen mobilized EPCs into circulation. Ovarectomized Tie2-GFP female mice (6-8 weeks of age) were implanted with 60 day slow release estradiol pellets one week pre-surgery. Histopaque density gradient isolated mononuclear cells were labeled with CD133 and CD133+ and GFP+ cells were analyzed through flow cytometry. The number (%) of EPCs in placebo receiving mice remained at base line (blue line) while supplementation of E2 significantly increased the % of circulating EPCs, which peaked on day 3 and returned to base-line by day 7.

Figure 5

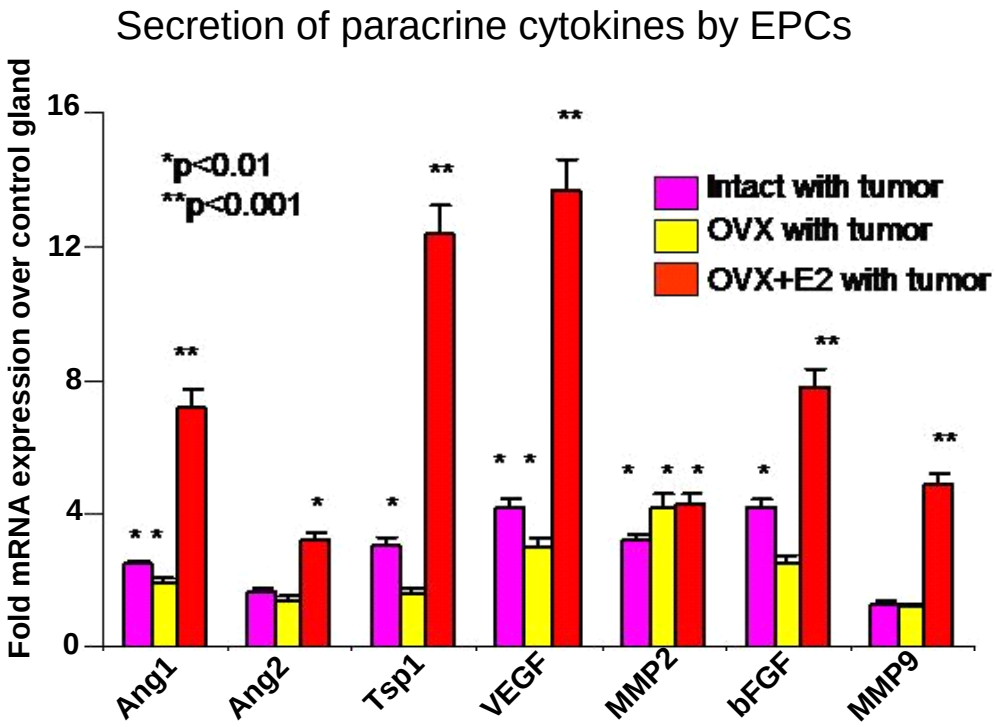

Figure 5. EPCs secrete paracrine angiogenic factors. Quantitative real-time PCR (Q-RT-PCR) analysis of mammary gland tissues reveal that E2 supplemented ovarectomized mice (OVX + E2) increase tissue remodeling and angiogenic cytokine mRNA transcripts compared to non-estrogen supplemented ovarectomized (OVX)

Figure 6

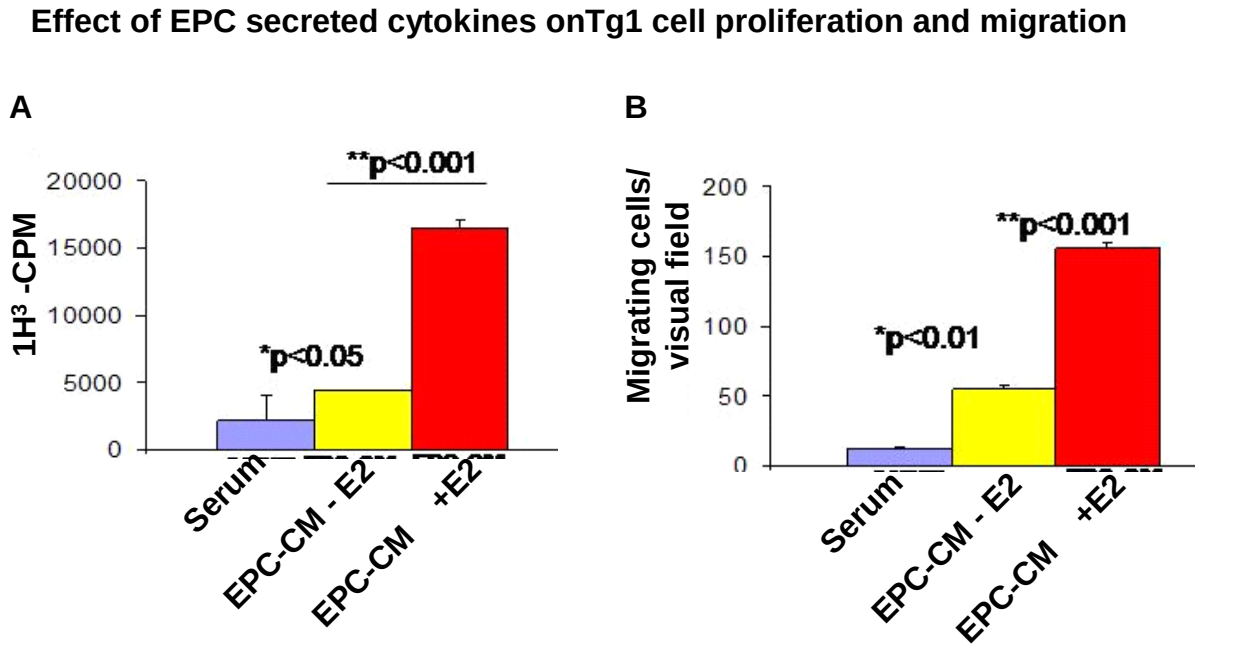

Figure 6. Estrogen stimulated EPCs secrete factors which induce tumor cell proliferation and migration. Supernatants of EPCs cultured in the presence of E2 secreted factors which stimulated the proliferation of 4T1 tumor cells (determined by tritiated thymidine (1H3-thymidine) incorporation (A) as well as increased 4T1 tumor cell migration as determined by using a using modified Boyden Chamber (B).

Figure 7

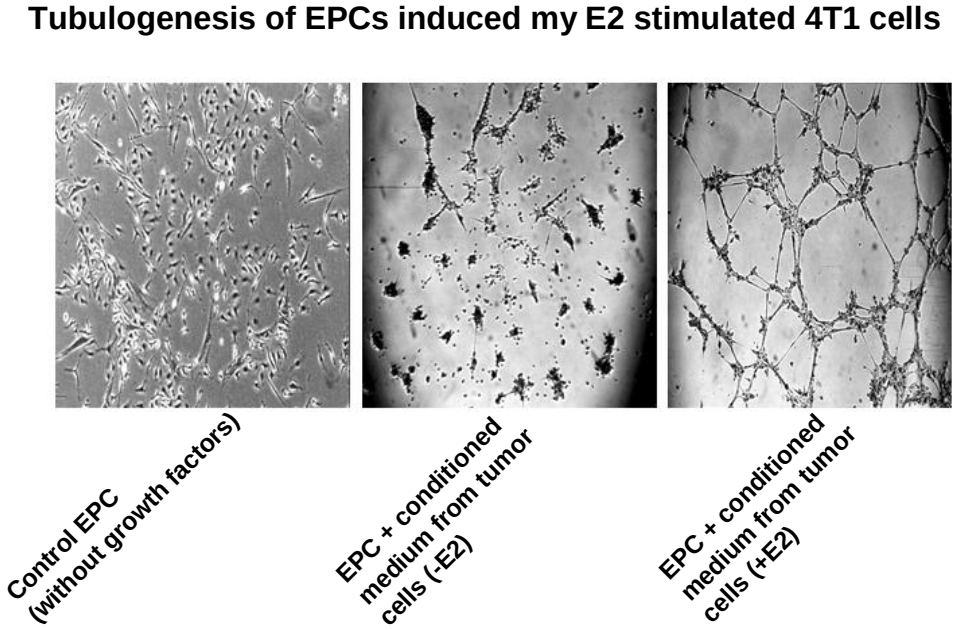

Figure 7. E2-induced factors secreted by tumor cell modulated EPC function through tubulogenesis. Addition of E2-treated tumor cell conditioned medium drastically enhanced EPC tubulogenesis with well-organized tubes observed as opposed to the few un-organized tubules present in non-E2 treated tumor cell medium.